Asia Pacific Journal of Molecular Biology and Biotechnology, 2007
Vol. 15(1) : 33-38

Trouble Shooting For Recalcitrant Bud Formation In *Capsicum Annuum* var. Kulai

Mok, S.H. and Norzulaani, K.*

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur.

Received 1 August 2006 / Accepted 10 December 2006

Abstract. Hypocotyls and cotyledons of *Capsicum annuum* var Kulai from 8-14 days old seedlings were cultured on MS medium supplemented with various concentrations of BAP and IAA. Effect of explant types, developmental stages of seedling and duration of subculture in different media were studied. Young tissue from hypocotyls formed more buds than the older tissue. Hypocotyls showed better response than cotyledons. Subculture was carried out every two weeks to prevent browning of explants and promote adventitious bud formation and development. Optimal bud forming efficiency in hypocotyl explants was obtained when explants were cultured on MS media containing 3 mg/l BAP and 0.3 mg/l IAA for 2 weeks followed by subculture on medium supplemented with 2mg/l BAP and 0.15mg/l IAA. The percentage of bud formation was 75.48 ± 4.71 %.

Keywords. Adventitious bud formation, *Capsicum annuum*

Chilli (*Capsicum annuum* L.) is among the most economically important fruits and vegetables worldwide. However, it is attack by many pathogens, including viruses, fungi, bacteria and nematodes (Tan *et al*., 1998; Arous *et al*., 2001). This has stimulated heavy use of chemicals to overcome pathogens.

The use of transgenic plants is a method to improve existing chilli cultivar. However, application of genetic engineering in chilli has been limited because of the difficulties in plant regeneration (Liu *et al*., 1990). Elongation of *in vitro* formed buds is a current problem in hot pepper (Gunay and Rao, 1978; Valera-Montero and Ochoa-Alejo, 1992) and sweet pepper varieties (Ebida and Hu, 1993; Zhu *et al*., 1996). It is however more predominant in the former than the latter.

In Malaysia, *in vitro* regeneration of several cultivars of chilli such as Langkap, MC 4, MC 5, chilli Bangi and Kulai had been studied (Marziah *et al*., 1995; Ahmad *et al*., 1997; Lim *et al*., 2000). Among these cultivars, Kulai is the most important in terms of economic value and area cultivated (Tan *et al*., 1998). However, there are limited reports available on Kulai and none of them provided promising response in plant regeneration of this cultivar.

As reported in most published papers, the ability of various pepper explants to regenerate in culture varies not only with the cell and tissues themselves but also with the species, cultivar and even the individual genotype (Ebida and Hu, 1993; Christopher and Rajam, 1994; Szász *et al*., 1995; Venkataiah *et al*., 2003). In addition, the developmental stage, location of plant tissue and environmental factors such as temperature, light regime and intensity are also critical in pepper shoot regeneration (Phillips and Hubstenberger, 1985; Arroyo and Revilla, 1991; Szász *et al*., 1995). This study evaluates some of the above-mentioned factors to optimize the best explant type and culture medium for *Capsicum annuum* var. Kulai.

Seeds of *Capsicum annuum* var. Kulai were obtained from the Malaysian Agricultural Research Development Institute (MARDI). To surface sterilized, seeds were soaked in 70 % ethanol (v/v) for one minute, followed by 20 mins in 20 % (v/v) commercially prepared bleach, (Clorox) (5.25 % active ingredient sodium hypochlorite) and a drop of surfactant, Tween 20 They were then rinsed thoroughly with sterile distilled water and blotted dry on sterile filter paper. Sterile seeds were germinated aseptically on MS basal medium (Murashige and Skoog, 1962).

Hypocotyls and cotyledons were taken from 8-14 day old seedlings according to experimental requirements. In selecting explants, physiological stages of development as well as the chronological age of the explants were taken into consideration. Young hypocotyls were excised from 8-9 day old seedlings at the hook stage where cotyledons were still enclosed inside the seed coat. Older hypocotyls were excised

*Author for Correspondence.
Mailing address: PBIU, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur. Tel: 603-79674380; Email:lani@um.edu.my

from upper part of hypocotyls from 12-14 day old seedlings. Young cotyledons were obtained from 10-12 day old seedlings with cotyledons still partially inside the seed coat while fully expanded cotyledons of 12-14 day old seedlings provided "old" cotyledons. All explants used were 3-4mm in length and cultured horizontally in MS basal medium with or without plant growth regulators.

After 2 weeks of culture, adventitious buds were subcultured onto the same or different media (depending on experimental requirements) for another 14 days. Then, buds (1-1.5cm) were transferred to elongation medium containing 2mg/l BAP, 0.15mg/l IAA and 2mg/l GA_3.

All culture media (MS supplemented with various concentrations of BAP and 3% (w/v) sucrose) were adjusted to pH 5.8 and solidified with 0.2% (w/v) phytagel before autoclaving at 121ÚC for 20 minutes. IAA was added into the media after autoclaving. Experiments were repeated at least three times and regenerative capacity of different explants was recorded based on the shoot bud formation. Subculture was carried out every 2 weeks to the same or different media depending on experimental requirement. All cultures were incubated at 25 ± 2ÚC with 16-h photoperiod from white cool lights.

Although formation of adventitious buds for some varieties of sweet and hot pepper was induced in hormonal-free medium (Ezura *et al*., 1993; Rafael Ramirez-Malagon and Ochoa-Alejo *et al*., 1996) and medium supplemented with only cytokinin (Ramage and Leung, 1996; Venkataiah *et al*., 2003), *Capsicum annuum* var. Kulai did not give similar response. In most of the protocols published to date, the most responsive hormonal medium uses IAA as auxin and BAP as cytokinin (Phillips and Hubstenberger, 1985; Agrawal *et al*., 1989; Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo, 1992; Christopher and Rajam, 1996; Mihalka *et al*., 2000). Thus, different combinations of BAP and IAA were used in our experiments to investigate the effect of these hormones on regeneration in different explant types.

Shoot buds appeared as a dark green ring in young and old hypocotyls after 8-9 days and 12-14 days respectively. Hypocotyl explants in culture grew to about 2-3 cm in length after 2 weeks of culture. Buds usually emerge from only one cut end, of the younger tissue. In the subculture medium, leaves developed from the adventitious buds. However, the shoot buds appeared abnormal after transfer into the elongation medium. The leaves became pale green and they broke readily at the point of attachment. Subsequently the buds turned brown and failed to elongate into a normal shoot (Figure 1). Cotyledons expanded to 2-3cm after a week of culture. The cut ends curled up towards each other and callus formed at the cut surfaces. The callus was white and yellow in colour. Adventitious buds from cotyledons also failed to develop further after 2 weeks in subculture medium.

Results in Table 1 show that adventitious bud formation depends on the type of explants, developmental stage, and concentration of hormones in the original and subculture medium. Hypocotyls and cotyledons responded differently in the medium tested. The percentage of bud induction ranged from 11.86 ± 3.53 to 63.99 ± 4.77 for hypocotyls and 16.03 ± 0.64 to 94.84 ± 3.68 for cotyledons. Frequency of bud formation was low when explants were cultured in medium containing 2mg/l BAP and 0.4mg/l IAA. Medium supplemented with 3mg/l BAP and 0.6mg/l IAA or 4mg/l BAP and 0.8mg/l IAA were found favorable for bud formation in both hypocotyls and cotyledons. In comparison to cotyledon explants, hypocotyls explants could produce developed buds although the former explants displayed a higher percentage of bud formation (Figure 1).

In this study, the best medium for young hypocotyls was 3mg/l BAP and 0.6mg/l IAA while 4mg/l BAP and 0.8mg/l IAA was the best combination for young cotyledons, old cotyledons and old hypocotyls. Highest percentage of bud formation for young and old hypocotyls was 63.99 ± 4.77% and 56.44 ± 1.89% respectively, while young cotyledons achieved 87.69 ± 4.19% and old cotyledons 94.84 ± 3.68%. It is therefore noted that slightly higher concentration of BAP was required for cotyledon explants compared to hypocotyl explants. Results suggest that old tissues and differentiated tissues required higher concentration of BAP.

The general trend showed that the percentage of bud formation decreased as the age of tissues increased with a few exceptions. This could be due to the competent cells in the older cotyledon which have higher totipotency. In general, younger tissues exhibited higher bud forming ability than older tissues in hypocotyls. Similar results have been reported by Ramirez-Malagon *et al*., (1996). They reported that seedlings at the curved hypocotyl stage (9 day old) were the explants that exhibited both the highest number of buds and shoots per explants compared to seedling at 15, 16, 21 and 28 days old.

Concentration of hormones in original and subculture medium also influenced bud and shoot formation. Usually, high cytokinin to auxin ratio is beneficial for bud regeneration (Phillips and Hubstenberger, 1985). In our experiment, when cytokinin to auxin ratio was increased in the subculture medium by raising the cytokinin concentration (but keeping IAA levels constant) it was found that the percentage of bud formation in hypocotyls decreased as BAP concentration increased (Table 1). However, there was no clear trend for cotyledon explants. Little growth and browning of explants occurred (reached as high as 40%) after 2 weeks of culture in higher concentration of BAP (Figure 2). Explants showed no further growth after subculture. Results indicate that explants have lost their competence to develop further after 4 weeks in culture. Prolonged culture in high concentration of BAP inhibited bud development and leaf expansion, hastened browning and caused abnormality of the explants. This is in congruence with the findings of Binzel *et al*., (1996). They reported that 10μM and 20μM cytokinin proved more beneficial in terms of normal elongation and development of buds, however, at higher concentration (30 μM) the shoot

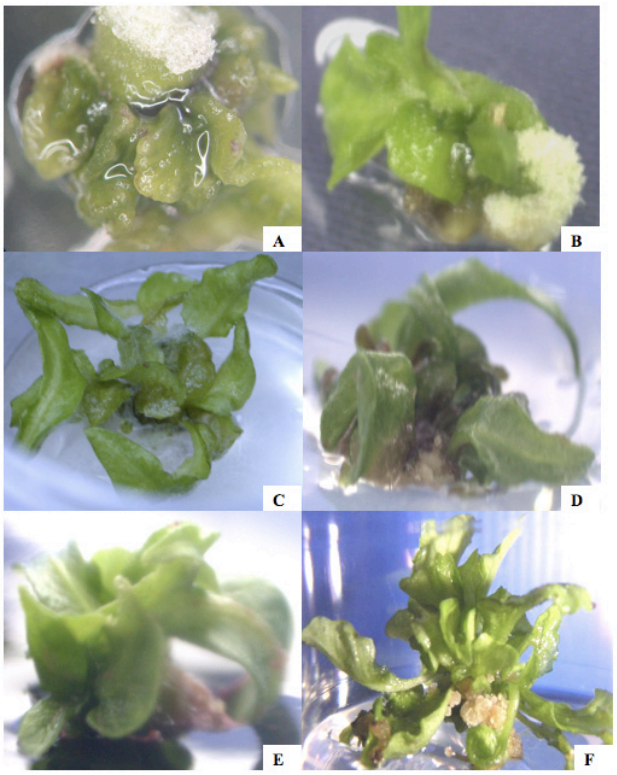

Figure 1. Adventitious bud formation on cut end of (A) cotyledon (4mg/l BAP + 0.8mg/l IAA) and (B) hypocotyl (3mg/l BAP + 0.3mg/l IAA) after 4 weeks in culture. C, D Leaf-like structure regenerating from hypocotyl explants E Buds were transferred to elongation medium, leaves broke readily at the point of attachment and failed to elongate into a normal shoot. F Formation of rosette-like shoot buds from hypocotyl explants in the elongation medium which containing 2 mg/l BAP, 0.15 mg/l IAA and 2 mg/l GA3.

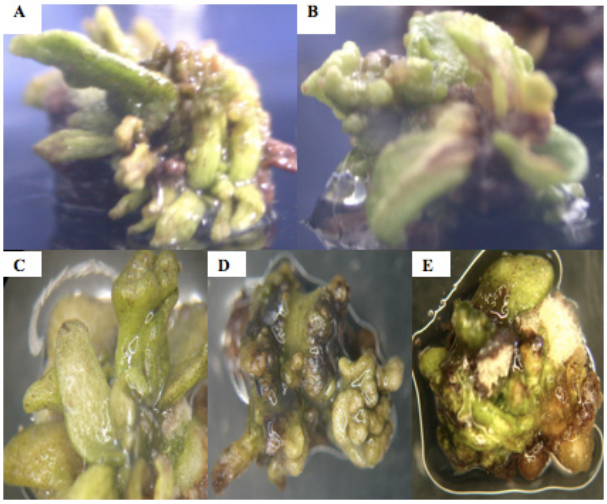

Figure 2. Browning of explants occurred after 2 weeks in subculture medium as BAP concentration increased A, B, C Bud development and leaf expansion was inhibited after 2 weeks of culture in higher concentration of BAP. D, E Bud turned brown and lost competence to develop further after 4 weeks in culture.

Table 1. Effect of culture medium and subculture medium on *in vitro* bud induction from different seedling explants

Original medium		Subculture medium		Percentage of bud formation (%)			
BAP (mg/l)	IAA (mg/l)	BAP (mg/l)	IAA (mg/l)	Young hypocotyl	Old hypocotyl	Young Cotyledon	Old Cotyledon
2	0.4	2	0.4	24.75 ± 2.53	20.20 ± 2.02	29.02 ± 1.75	19.45 ± 2.78
		4	0.4	20.63 ± 2.45	24.36 ± 8.98	16.03 ± 0.64	56.67 ± 1.5
3	0.6	3	0.6	63.99 ± 4.77	41.31 ± 2.99	48.71 ± 4.13	34.19 ± 3.19
		5	0.6	29.16 ± 5.15	14.65 ± 3.54	47.21 ± 2.01	94.84 ± 3.68
4	0.8	4	0.8	45.92 ± 0.33	56.44 ± 1.89	87.69 ± 4.19	71.47 ± 3.52
		6.5	0.8	34.19 ± 3.20	22.41 ± 2.05	81.24 ± 1.99	52.80 ± 1.99
5	1.0	5	1.0	49.98 ± 5.91	25.18 ± 2.08	58.33 ± 3.40	67.46 ± 2.97
		10	1.0	32.05 ± 1.28	11.86 ± 3.53	48.31 ± 3.56	22.05 ± 4.33

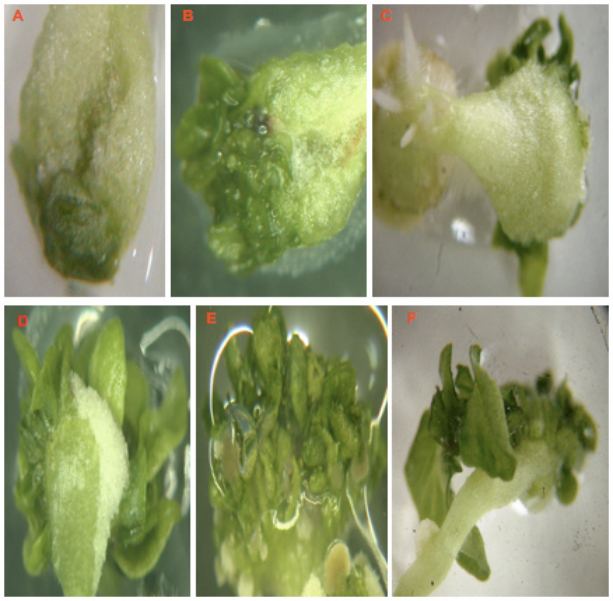

Figure 3. In vitro bud formation from hypocotyls explants (A) a dark green ring at cut ends of hypocotyls (B) buds growing at cut ends of hypocotyls (C and D) adventitious buds developed at cut ends of hypocotyls (E) a cluster of small, compact and rosettes buds after 4 weeks in culture (F) hypocotyls with developed leaves without shoot axis was visible.

Table 2. Percentage of bud formation (%) for hypocotyl explants on different treatment

Original medium		Subculture medium		Percentage of bud formation (%)
BAP (mg/l)	IAA (mg/l)	BAP (mg/l)	IAA (mg/l)	
3	0.6	3	0.6	48.91 ± 6.07
		3	0.3	67.42 ± 4.41
		2	0.3	72.78 ± 3.74
3	0.3	3	0.3	53.67 ± 4.51
		3	0.15	66.29 ± 4.50
		2	0.15	75.48 ± 4.71

buds remained extremely small and leaves became quite thick and ill-defined.

The influence of concentration and balance of cytokinin and auxin in the subculture medium on bud initiation and development were studied. This experiment was conducted using hypocotyl explants. A high cytokinin to auxin ratio was obtained by maintaining the BAP concentration and reducing the IAA concentration, or by decreasing both the concentration of BAP and IAA. Results obtained show that 8-9 day old hypocotyls cultured in 3mg/l BAP and 0.3mg/l IAA exhibited the dark green ring after 8-9 days (Figure 3). Hypocotyls in 3mg/l BAP and 0.6mg/l IAA required 10-14 days to show dark green ring. Our results suggest that an extremely critical concentration and delicate balance of cytokinin and auxin is effecting bud formation.

As shown in Table 2, a slightly higher percentage of bud formation was consistently recorded for hypocotyls cultured in 3mg/l BAP and 0.3mg/l IAA than in medium containing 3mg/l BAP and 0.6mg/l IAA. Explants cultured on 3mg/l BAP and 0.6mg/l IAA and subcultured to the same treatment consistently give lowest production of buds. Bud formation was enhanced when the explants were subcultured to medium containing same concentration of BAP but reduced concentration of IAA. The highest percentage of bud formation was obtained in subculture medium where concentrations of both BAP and IAA were reduced. A similar trend was observed for explants in 3mg/l BAP and 0.3mg/l IAA. Explants without subculture exhibited callus formation and a slower rate of bud induction. Very often no buds were observed after 4 weeks.

In general, it is preferable to subculture to a lower concentration of BAP and IAA medium provided cytokinin to auxin ratio in the subculture medium is higher than in the original medium. There was no browning of explants observed using this protocol and most of the buds developed further during subculture. The percentage of bud formation was increased in this experiment. Although the percentage bud formation in hypocotyl explants in 3mg/l BAP and 0.3mg/l IAA and 3mg/l BAP and 0.6mg/l IAA is similar (Table 2), nevertheless, consistently higher values are obtained in 3mg/l BAP and 0.3mg/l IAA. Hence, in the present study the best bud forming efficiency has been repeatedly achieved when hypocotyl explants are cultured on 3mg/l BAP and 0.3mg/l IAA for 2 weeks followed by subculture on 2mg/l BAP and 0.15mg/l IAA. However, further test could be carried out in order to obtain better results.

Often, bud formation was not translated to shoot development/plant regeneration. Regeneration of pepper plants via organogenesis is severely limited because of the difficulties in efficient development of induced buds into complete plants (Liu *et al.*, 1990; Szász *et al.*, 1995). In many cultivars, the shoot buds either do not elongate or produce aberrant, ill-defined, distorted leaves (Gunay and Rao, 1978; Arroyo and Revilla, 1991; Valera-Montero and Ochoa-Alejo, 1992; Ebida and Hu, 1993). Same difficulties were experienced in our present study and infrequent *in vitro* shoot elongation was obtained. Attempts to elongate these shoot buds, such as culture in low BAP and IAA (Phillips and Hubstenberger, 1985; Christopher and Rajam, 1994; Venkataiah *et al.*, 2006); giving explants a liquid cytokinin pulse (Goldfarb *et al.*, 1991) and addition of GA_3 or $AgNO_3$

were unsuccessful.

In conclusion, our results suggest that type of explants, physiological age/ development stage of explants influence the percentage of bud and shoot formation. In addition, cytokinin to auxin ratio, their concentration in culture and subculture media, duration of culture and subculture are crucial determinants in the induction of buds and shoot formation. There were differences in bud forming ability between young and old tissue. Young tissue shows better response than older tissue in the different media tested. Adventitious buds tended to develop further when explants were subcultured in cytokinin and auxin concentration that is lower than that in the original medium. Subculture every two weeks is recommended in order to obtain higher percentage of bud formation and avoid browning of the explants.

Acknowledgement

Authors would like to thank Dr. Tung, H. F. for assistance in this study and the Ministry of Science, Technology and the Environment for the generous grant (IRPA 31-02-03-1043). This study also supported by the University of Malaya (Vote F 0171 / 2005C).

References

Agrawal, S., Chandra, N. and Kothari, S. L. 1989. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L. cv. Mathania). *Plant Cell, Tissue and Organ Culture*. 16: 47-55.

Ahmad, I.B., Zaiton, A., and Zarina, S. 1997. Transformation of *Capsicum annuum* with *Agrobacterium*. 9th National Biotechnology Seminar. p.g. 127-130.

Arous, S., Boussaid, M. and Marrakchi, M. 2001. Plant regeneration from zygotic embryo hypocotyls of Tunisian chili (*Capsicum annuum* L.). *Journal Appl. Hort.* 3(1): 17-22.

Arroyo, R. and Revilla, M. A. 1991. *In vitro* plant regeneration from cotyledon and hypocotyls segments in two bell pepper cultivars. *Plant Cell Reports*. 10: 414-416.

Binzel, M. L., Sankhla, N., Joshi, S. and Sankhla, D. 1996. *In vitro* regeneration in chile pepper (*Capsicum annuum* L.) from 'half-sed explants'. *Plant Growth Regulation*. 20: 287-293.

Christopher, T. and Rajam, M. V. 1994. *In vitro* clonal propagation of *Capsicum* spp. *Plant Cell, Tissue and Organ Culture*. 38: 25-29.

Christopher, T., Rajam, M. V. 1996. Effect of genotype, explant and medium on *in vitro* regeneration of red pepper. *Plant Cell, Tissue and Organ Culture*. 46: 245-250.

Ebida, A. I. A. and Hu, C. Y. 1993. *In vitro* morphogenetic responses and plant regeneration from pepper (*Capsicum annuum* L. cv. Early California Wonder) seedling explants. *Plant Cell Reports*. 13:107-110.

Ezura, H., Nishimiya, S. and Kasumi, M. 1993. Efficient regeneration of plants independent of exogenous growth regulators in bell pepper (*Capsicum annuum* L.). *Plant Cell Reports*. 12: 676-680.

Fari, M. and Czako, M. 1981. Relationship between position and morphogenetic response of pepper hypocotyls explants cultured *in vitro*. *Scientia Horticulturae*. 15:207-213.

Goldfarb, B., Howe, G. T., Bailey, L. B., Strauss, S. H. and Zaerr, J. B. 1991. A liquid cytokinin pulse induces adventitious shoot formation from Douglas-fir cotyledons. *Plant Cell Reports*. 10: 156-160.

Gunay, A. L., Rao, P. S. 1978. *In vitro* plant regeneration from hypocotyls and cotyledon explants of red pepper (*Capsicum*). *Plant Science Letter*. 11: 365-372.

Lim, J. H., Loh, S. T., Tan.C. S. 2000. Expression of reporter gene in chilli plant using *Agrobacterium*-mediated transformation. 12th National Biotechnology Seminar. p.g. 431-433.

Liu, W., Parrot, W. A., Hilderbrand, D. F., Collins, G. B. and Williams, E. G., 1990. *Agrobacterium* induced gall formation in bell pepper (*Capsicum annuum* L.) and formation of shoot like structures expressing introduced genes. *Plant Cell Reports*. 9: 360-364.

Marziah, M., Zaliha, A. and Kow, C. W. 1995. Studies of tissue culture of different chilli cultivars (*Capsicum annuum* L.). *Asia Pacific Journal of Molecular Biology and biotechnology*. 3(2): 96-105.

Mihalka, V., Fari, M., Szasz, A., Balazs, E. and Nagy, I. 2000. Optimised protocols for efficient plant regeneration and gene transfer in pepper (*Capsicum annuum* L.). *Journal of Plant Biotechnology*. 2(3): 143-149.

Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays of tobacco tissue cultures. *Physiol. Plant*. 15: 473-497.

Phillips, G. C. and Hubstenberger, J. F. 1985. Organogenesis

in pepper tissue cultures. *Plant Cell Tissue Organ Culture*. 4: 261-269.

Ramage, C. M. and Leung, W. M. 1996. Influence of BA and sucrose on the competence and determination of pepper (*Capsicum annuum* L. var. Sweet Banana) hypocotyls cultures during shoot formation. *Plant Cell Reports*.15: 974-979.

Ramirez-Malagon, R. and Ochoa-Alejo., N. 1996. An improved and reliable chilli pepper (*Capsicum annuum* L.) plant regeneration method. *Plant Cell Reports*. 16: 226-231.

Szasz, A. Nervo, G. and Miklos, F. 1995. Screening for in vitro shoot-forming capacity of seedling explants in bell pepper (*Capsicum annuum* L.) genotypes and efficient plant regeneration using thidiazuron. *Plant Cell Reports*. 14: 666-669.

Tan, C.S., Ong, C. A. and Hassan, M.D. 1998. Cloning and expression of *cucumber mosaic virus* coat protein in bacteria. 10th National Biotechnology Seminar. p.g. 40-46.

Valera-Montero, L.L. and Ochoa-Alejo, N. 1992. A novel approach for chili pepper (*Capsicum annuum* L.) plant regeneration: shoot induction in rooted hypocotyls. *Plant Science*. 84: 215-219.

Venkataiah, P., Christopher, T. and Subhash, K. 2003. Thidiazuron induced high frequency adventitious shoot formation and plant regeneration in *Capsicum annuum* L. *Journal of Plant Biotechnology*. 5(4): 245-250.

Venkataiah, P., Christopher, T. and Subhash, K. 2006. *In vitro* shoot multiplication and plant regeneration in four *Capsicum* species using thidiazuron. *Scientia Horticulturae*. 107: 117-122.

Zhu, Y. X., Ou-Yang, W.J., Zhang, Y. F. and Chen, Z.L. 1996. Transgenic sweet pepper plants from *Agrobacterium mediated* transformation. *Plant Cell Reports* 16: 71-75.